

Characterization of Torque Teno Virus by *In Vitro* Infection of Gnotobiotic Pigs:  
Torque Teno Virus the Cause of PAS?

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## Abstract

Viruses are important disease causing agents prevalent in all animal species. Understanding their characteristics and pathogenicity are crucial to control and prevent disease. Piglet Anemia Syndrome (PAS) is a neonatal anemia in piglets with no known cause. We began this project by looking at the possibility that porcine torque teno virus (TTV) plays a role in causing PAS disease syndrome. Chapter 1 characterizes genotype(s) of TTV identified in a continuous porcine alveolar macrophage cell line. Chapter 2 delineates preliminary in vivo challenge experiments with this TTV genotype.

The porcine TTVs are classified into two genogroups (g1-TTV and g2-TTV). Like other *Anelloviruses* genomic diversity is a hallmark of porcine TTVs. Porcine TTV was found in a porcine alveolar macrophage cell line (3D4/2) and sequenced for identification and classification (Chapter 1). The macrophage cell line was then used to infect gnotobiotic piglets in order to determine its infectious capabilities (Chapter 2). The CD4/31-origin TTV used for the *in vivo* infection experiment was found to have an 81 percent homology to a combined g1- and g2-TTV sequence indicating the possible presence of a hybrid TTV virus. On or before ten days after challenge infection, in gnotobiotic pigs were shown to be TTV DNA viremic. In the challenged piglets, neither gross nor histologic findings characteristic of TTV infection were observed. These experiments were performed with the goal of obtaining knowledge that could potentially lead to the development of vaccines and reduce the presence of PAS in pig populations.

## Background

**Chick Anemia Virus (CAV) as a model of Anellovirus-associated Anemia:** Many viral infections affect bone marrow cells as a part of their lytic replication cycle. For example, the Parvoviridae (double-stranded nonenveloped DNA viruses) have affinities for rapidly dividing cells. Feline panleukopenia virus (FPV) is well known for its often lethal effects in young cats. However, only a few nonretroviral viruses replicate in bone marrow erythrocyte precursor stem cells and induce a life-threatening nonregenerative anemia in infected animals. Of these, chick anemia virus (CAV), a *Gyrovirus* in the Circoviridae family, is the most widely studied and characterized (fig. 1). It was first described in day-old specific-pathogen-free chicks that developed fatal aplastic anemia caused by a filterable (viral) agent. CAV infection in older birds also replicate in bone marrow cells but do not cause sufficient cell loss(es) to result in anemia. CAV infection of adult birds (layers) can cause immune suppression in adults thereby reducing performance. CAV is transmitted from subclinically infected hens to *in ovo*-developing fetuses and, depending upon the degree of bone marrow cell destruction, result in the hatching of severely anemic chicks. Vaccination(s) of hens induce adequate maternal humoral immunity thereby protecting the chicks during their first two to three weeks of life.

**The potential role of porcine torque teno viruses in the piglet anemia syndrome:** Other members of the Circoviridae family include members of the *Circovirus* genus (the porcine circoviruses types 1 and 2 and other avian pathogens) and the *Anellovirus* genus (the species specific torque teno virus (TTV) and torque teno mini viruses (TTMVs). The Circoviridae family

has very small DNA viral pathogens characterized by a unique form of viral DNA, circular and single-stranded. The genomes are small (1800-4000 bases) and contain genetic information for only two significant proteins: a virus-specific DNA replicase (rep) and a viral nucleocapsid protein.

**PIGLET ANEMIA SYNDROME:** Piglet Anemia Syndrome (PAS) is a newly discovered disorder of



Figure 2: PAS often affects suckling pigs (above)

weaning and suckling pigs (fig.2) and is characterized chiefly as a neonatal anemia syndrome. Inspection of hemograms indicate that affected piglets are pancytopenic and thrombocytopenic, likely a reflection of a

widespread defect in bone marrow stem cells, particularly the stem cell that gives rise to platelets and erythrocytes. Piglets are unresponsive to colloidal iron injections for symptomatic treatment for anemia and develop a “failure to thrive” syndrome, increased peri-natal mortality and are eventually culled from the herd as poor-doers.

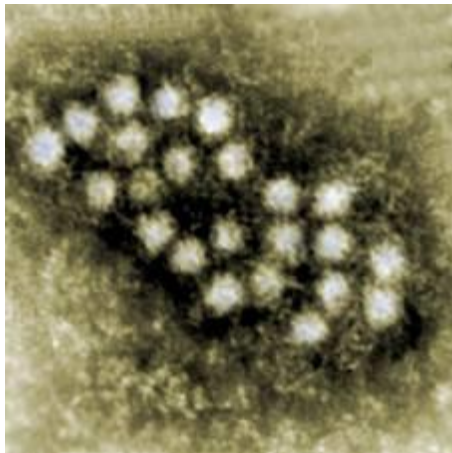
The cause of PAS is not known; all of the usual causes of piglet anemias (iron deficiency, maternal-origin isoantibodies to piglet erythrocytes (analogous to Rh factor anemia in humans), diet, genetics of sow/boars used environmental toxins and poisons and intercurrent infections by other swine viral and bacterial pathogens) have been eliminated as casual factors in PAS. PAS tends to affect herds that are very healthy (High herd health profiles), are well vaccinated

and controlled for common porcine infectious agents such as *Mycoplasma hyopneumoniae* (porcine respiratory disease complex), porcine reproductive and respiratory syndrome (PRRS) virus, porcine circovirus type 2 (PCV2) and swine influenza virus (SIV). Aside from the anemia syndrome in young pigs, very little is known about how PAS affects the health of weanling, feeding and adult swine populations. Piglets with PAS may be born pale or stillborn and in some cases piglets will become pale within their first week of life. The syndrome may cause a mild decrease in white blood cells, including neutrophils and lymphocytes, a decrease in platelets, and a reduced volume of red blood cells. The red blood cell count in these piglets may be 5 – 27 % less than in control piglets. Tissue examination(s) for the known porcine pathogens in PAS piglets are negative. There is also no evidence of increased erythrocyte fragility or intravascular hemolysis that would indicate autoimmune hemolytic anemia or toxin and drug-mediated bone marrow destruction. In neonatal anemia in pigs, a common problem is iron deficiency but in the case of PAS pigs this does not seem to be the problem as the anemia of PAS cannot be improved by iron supplementation. The only other organs that may be affected are the liver and spleen that exhibit extramedullary hematopoiesis (EMH). EMH is a physiological response to anemia and is more severe the older the animal becomes. PAS becomes more severe as the infected animal ages because of its decreasing ability to transport oxygen to tissues.

In almost all cases where piglets have signs of PAS, sows are clinically normal. The incidence of PAS varies within a litter from none to over 50 % and the number of litters a sow

has in her lifetime has no effect on the presence of PAS. PAS does not seem to be dependent on the season or time of year and it does not seem to be genetically inheritable from either the sow or boar. Veterinarians and herdsman are at a loss to explain the causation of PAS other than to note that the disease pattern(s) in affected herds is most compatible with an unknown or undiagnosed viral infection.

**A POTENTIAL VIRAL CANDIDATE FOR PAS IS THE PORCINE TORQUE TENO VIRUSES (TTVs):** The TTVs are species-specific and infect many species including humans and animals (fig. 3). In humans, the presence of the TTV virus is not yet directly associated with diseases although high



levels of circulating TTV DNAs are correlated to patients with asthma and autoimmune diseases. It is possible TTV infection can facilitate different disease conditions as co-infections.

Swine possess their own TTVs. Sequence analyses have delineated two genogroups of porcine TTVs,

**Figure 3: Electron microscopy of TTV**

genogroups (g) 1 and 2. Porcine TTVs are thought to be

similar to those found in humans and are also thought to be nonpathogenic when no other infections are present. However, recent work at OSU has conclusively demonstrated that porcine TTVs are pathogens in gnotobiotic (germfree) pigs ("Krakowka, Allan, Ellis", 2008).

Further, *in utero* transmission of TTVs (from virus-positive sows to developing piglets) is established as one mode of transmission of the porcine TTVs. Porcine TTVs replicate in bone

marrow cells within infected piglets and the virus appears to have affinities for bone marrow stem cells and monocytoïd precursor cells. The porcine (and human) TTVs are currently unculturable *in vitro*. The only method of detection of TTV infection is by PCR methodologies. The TTVs are an excellent candidate virus(es) for the etiology of PAS. The virus replicates in bone marrow stem cells and macrophage lineage cells and viral cytopathology appears to be associated with virus-induced apoptosis in this cell population. As well, the TTVs are genetically diverse and it is likely that only a few genotypes within either genogroup 1 or 2 induce PAS. Important gaps in our knowledge regarding PAS are the nature of the infectious agent and the mechanism whereby the agent of PAS is transmitted to piglets. Since the variables that contribute to PAS are not completely understood, analysis of TTV transmission could provide additional insights into PAS. It has been documented that *in utero* infection is one important mode of TTV transmission. This process however, does not make TTV transmission predictable in pigs in the postnatal environment. The incidence of *in utero* transmission is litter-dependent. This indicates that some litters may contain no TTV infections while others can contain as much as a 20 % transmission rate. It has also been shown that a TTV-negative sow may give birth to TTV positive piglets. This could be the result of a protective immunity in the sow after fetal infection occurred. As PAS continues to be studied, more importance is placed on the analysis and comparison to non-PAS genotypes of TTV.

By analogy of CAV in poultry, the TTVs are strong candidate viral agents for the etiology of PAS. CAV is a close virologic relative to TTVs and is known to cause aplastic anemia in chicks. It is likely that these two viruses share at least some of the same biological properties in the



host species properties. Porcine TTV as a cause of disease has been delineated in gnotobiotic piglets. The published paper, *Evaluation of the effects of porcine genogroup 1 torque teno virus in gnotobiotic swine* determined if genogroup 1 TTV (g1-TTV) has the ability to infect and cause disease in gnotobiotic swine. In all cases pigs infected with g1-TTV had no clinical signs of infection but they did suffer from pneumonia, transient thymic atrophy, membranous glomerulonephropathy, and modest lymphocytic to histiocytic infiltrates in the liver. Based on these results it can be concluded that g1-TTV can readily infect gnotobiotic swine and infection is associated with characteristic pathologic changes. Therefore, a g1-TTV infection could cause an unrecognized pathogenic viral infection. The fact that TTVs can be present and undetectable in commercial swine populations makes it a potentially dangerous and a newly emergent infectious disease problem in swine.



**Figure 4: Piglets diagnosed with PMWS**

A link has been made between g1-TTV and the infection of PCV2 in Postweaning Multisystemic Wasting Syndrome (PMWS) (fig. 4). PMWS is a viral infectious disease in pigs and is found in all areas of the world. The necessary cause of PMWS is porcine circovirus type 2, also a member of the Circoviridae family. PMWS affects the pulmonary, enteric, and renal systems and can be lethal in populations of pigs. The side-effects are persistent fever, anorexia, progressive weight loss and death attributable to other bacteria and viruses found in swine. Even if pigs survive PMWS they have poor weight gain and are economically detrimental to swine producers. Many studies have evidenced that PMWS is caused by PCV2. This is furthered demonstrated by the decrease and/or elimination

of PMWS when an inactivated PCV2 vaccine is used in PMWS-affected herds. However, historically veterinarians are skeptical about this correlation due to the high incidence of subclinical PCV2 infection found in clinically normal pigs. This fact indicates that there must be a cofactor or amplifiers to fully express the disease and some experiments have indicated this. Infection with porcine g1-TTV appears to amplify the affects of PMWS in PCV2 infected pigs. This indicates that TTVs can be added to the group of infectious agents that can promote the infection of PMWS. Whether or not g1-TTV can amplify the PCV2 infection is dependent on the order of co-infection. If PCV2 is infectious in pigs before g1-TTV then amplification does not occur whereas, infection of g1-TTV prior to the infection of PCV2 facilitates the full expression of the PCV2 infection about 50 % of the time. The evidence that TTV has the ability to amplify the disease caused by other common porcine infectious disease viruses makes it critical to further study how TTV contributes to other diseases like PAS. The sole infection of pigs with TTVs has little clinical effects on swine populations. Due to the fact that TTVs are thought to be closely associated with PAS it can be hypothesized that PAS may have a similar affect as a co-factor in other porcine diseases. This will be important to keep in mind as PAS is further studied.

The purpose of this research is twofold: firstly to further characterize and clone a potentially new porcine TTV that has been found in porcine alveolar macrophage cell line(s). This was achieved by isolating the viral DNA, amplifying it by way of polymerase chain reaction (PCR) and sequence the DNA sample. This sequence is then compared to other closely related

TTVs. Secondly, we wished to determine if this *in vitro*-origin (3D4/2 cells) TTV is infectious by challenge infection into TTV-DNA-negative gnotobiotic swine. The overall goal of this second experiment is to challenge gnotobiotic pigs with this *in vitro*-propagated TTV sequenced virus, and upon piglet termination extract the virus from tissue samples, reconfirm its presence and identify infectious symptoms caused by this porcine virus.

## **Chapter: 1 – Cloning and Sequencing of 3D4/2-origin TTV**

**Section: 1.1 – Overview** TTV is a negative sense single stranded circular DNA virus (approx. 3,800 nucleotides) and is highly susceptible to mutations in the translated region of its genome (Hedman et al., 2009). PCR is the only known way to identify TTV and it was first identified using primers designed to attach on the conserved nontranslated region of its genome. Many sequence-distinct genotypes of the torque teno virus were identified indicating its wide genetic variability. In humans, TTV is known to have a genetic variability of up to 30% in a group of 39 genotypes and up to 50% genetic variation between five major genetic groups (Okamoto, 2009). It is unknown how TTV replicates in cells, however most other single stranded DNA viruses utilize the rolling circle mechanism, and it is thought that the TTV genome encodes two proteins (nucleocapsid protein and the viral DNA replicase protein) (Hedman et al., 2009). A DNA polymerase is not a part of the TTV genome; the virus must utilize host cell polymerase proteins in order for replication to occur.

By utilizing qPCR techniques porcine TTV was discovered in a porcine alveolar macrophage (3D4/2) cell line from the American Tissue Culture Collection (ATCC, catalog number CRL-2845). These cells contained g1- and g2-TTV viral DNAs as determined by Susan Ringler (personal communication). The objective of this study was to genotype the virus using DNA sequencing procedures. This would not only allow the virus to be specifically identified but would also allow the comparison of the specific strain of TTV to symptoms exhibited by gnotobiotic pigs (Section: 2.2).

## Section: 1.2 – Methods and Procedures

**STARTING MATERIAL: 3D4/2 CELL LINE:** The pigs were infected with a cell line designated: 3D4/2 containing g1- and g2-TTV viral DNAs. These cells were obtained from the American Type Culture Collection (ATCC), catalog number: CRL-2845. Upon receiving the cells, lab technician, Susan Ringler, subcultured the cell line. The cells were grown as suspension and loosely adherent cultures in RPMI-1640 medium with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum. The cell line derived from porcine lung macrophages (alveolar) and had been transformed with pSV3-neo (Weingartl, 2002). The cells were propagated at 37°C in a 5% CO<sub>2</sub> Incubator.

**CLONING OF TTV DNA:** The first step in sequencing a strain of virus is to isolate its' DNA. This was accomplished by first extracting DNA from the 3D4/2 cell line. The cell pellet was resuspended in PBS (phosphate-buffered saline) and DNA isolation was achieved with the Quiagen DNeasy Blood and Tissue Kit. A portion of cellular fluid was removed from the cells contained in the culture media and suspended in sterile water. This suspension contained a combination of viral and cellular DNA. Viral DNA was then amplified using PCR and either g1-TTV specific primers (g1-TTV forward: 5'-GCG GTC AAA ATG GCG GAA GG-3'; g1-TTV reverse: 5'-GGA CTT GAG CTC CCG ACC AA-3') or g2-TTV specific primers (g2-TTV forward: 5'-AGT AAG TGC GCA GAC GAA TGG-3' ; g2-TTV reverse: 5'-CCC AGG CGG TTA GAC ACT CA-3'). Following PCR, the amplified viral DNA was separated from the cell chromosomal DNA by gel electrophoresis. Upon conclusion of the electrophoresis multiple bands were visualized in each well. The DNA

band that represented the viral DNA segment (approx. 200 bp) was then cut out of the gel using a scalpel and placed into a microcentrifuge tube (fig. 5). The DNA in the gel fragment was

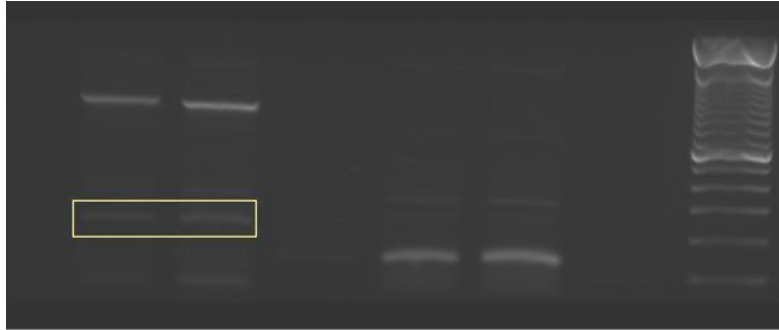


Figure 5: g1-TTV viral DNA band found in gel electrophoresis

subjected to purification by eliminating the gel and resuspending the viral DNA in solution using a Gel Purification Kit (Quiagen, QIAquick Gel

Extraction Kit). The DNA was then cloned by inserting the segment into a TOPO plasmid (Invitrogen). The recombinant TOPO plasmids were then used to transform *E. coli* (Top10). The bacteria were plated onto LB agar containing ampicillin and individual colonies were selected for further analysis. Plasmids containing copies of the viral DNA were then purified using a miniprep plasmid extraction kit (Promega). The purified plasmids which contained viral DNA inserts were sent to the Plant Microbe Genetics Facility at The Ohio State University for sequencing. This entire procedure was executed twice

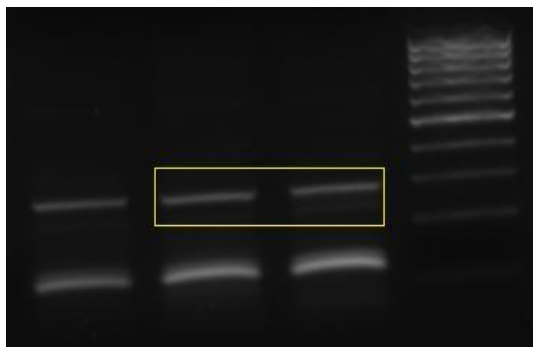


Figure 6: g2-TTV viral DNA band found in gel electrophoresis

for two different isolated bands. The first band was expected to be viral g1-TTV (fig. 5). The second time the procedure was executed g2-TTV specific primers were used and a DNA band at the expected g2-TTV length was cut and purified (fig. 6).

**SEQUENCE ANALYSIS OF CLONED TTV:** The sequenced segments of viral DNA were analyzed using Geneious, a DNA alignment software program. The plasmid sequences were identified and separated from the viral DNA sequences. A BLAST search using the GenBank database was performed. The insert and plasmid that was sequenced contained 1,405 base pairs (bp) in the g1-TTV sample and 1,355 bp in the g2-TTV sample. The TOPO plasmid restriction sites, which were on either side of the insertion region, were identified and the cloned segment of viral DNA was identified from the plasmid using the Geneious software. The cloned TTV DNA was 173 bp in length for the g1-TTV segment and 243 bp in length for the g2-TTV segment, which matched the size of both the designed primers. The highest percentage match with known sequences of TTV exhibited by cloned 3D4/2 g1-TTV viral DNA was 55%, while the cloned g2-TTV viral DNA did not align with any known TTV sequences. The cloned g2-TTV did however align by a 99.5 percent homology to pig DNA sequence from clone CH242-347M1 on chromosome 12. This was discovered from a BLAST search in the Geneious software using the cloned g2-TTV DNA as a template to identify all similar known nucleotide sequences. The percent homology of both sequence searches indicate the percentage of pairwise residues that are identical in the alignment, including gap vs. non-gap residues, but excluding gap vs. gap residues.

By PCR, the 3D4/2 cell line used to clone a g1- and a g2-TTV-like sequence is known to contain both genogroups 1 and 2 TTV. The Geneious software was again used to compare the g1-TTV 3D4/2 cell-origin cloned viral sequence to known sequences of g1- and g2-TTV using the Genbank database. When matched with both g1- and g2-genogroups, the percent homology rose from 55 to 81% (figure 7).

**Section: 1.3 – Discussion:** We will first discuss the significance and relevance of the cloned viral g1-TTV DNA related to known TTV sequences. Our g1-TTV nucleotide sequence matched known TTV sequences by a maximum of 55 percent. Some important characteristics of TTV must be taken into account before analysis of such a low percentage match can be properly discussed. TTV has been discovered and isolated in a wide variety of tissues including: lung, blood mononuclear cells, bone marrow, liver, lymph node, thyroid gland, spleen, pancreas, and kidney giving TTV a wide host cell tropism (Hedman et al., 2009). Since the TTV mechanisms and location of replication are unknown, other single stranded DNA viruses are used in comparison to possibly establish a rough sequence of events. All other single stranded negative sense DNA viruses must go through a DNA duplication phase in which DNA polymerase duplicates the single stand into a double stranded (ds) DNA. In order for the viral proteins to be utilized, a second DNA dependent RNA polymerase protein must then unzip the double stranded DNA and replicate messenger RNAs (mRNA) for protein synthesis. Finally the most important part of viral replication is reproducing copies of the original viral genome by polymerizing the negative sense DNA strand using DNA polymerase on the positive sense strand. Therefore a single stranded viral DNA molecule must go through three separate replications in order to achieve duplicated viral genome to reassemble entire viruses for further infection in nearby cells. This process utilized by other single stranded DNA viruses requires the viral genome to be replicated three times (once into a ds DNA molecule, once into mRNAs, and finally into its original negative sense strand from the positive sense strand). Replicating a viral genome three times gives a much greater probability that replication mistakes and/or mutations may arise. Therefore if TTV follows this general rule established by all other ss DNA



viruses it too has a higher probability for replication mistakes and/or mutations. Another interesting characteristic of TTV is that it mutates at a high frequency compared to many other viruses. Mutations can occur in a number of ways (e.g. substitutions, additions, deletions etc.) however, these are often avoided in cells by the proofreading mechanisms employed by the polymerase proteins. It has been discovered that some known virus genomes encode polymerase proteins that do not exhibit these proofreading mechanisms (namely HIV). TTV does not encode its own polymerase and most likely uses the cells polymerases that would be expected to have these proofreading characteristics. The question then becomes why does TTV mutate so much if the DNA polymerase is not to blame?

We can now return to the original question of why our TTV had such a low percent match to all other known g1-TTV genomes. The 3D4/2 cell line had been identified to contain both genotypes of porcine TTVs (g1- and g2-TTV). Both of these TTVs are separate genotypes of a genogroup and both likely originated from a single archival TTV genome. The fact that both are replicating simultaneously in the 3D4/2 cell line suggest that it is probable that they have similar replication steps and that they were occurring at the same place and time. Given the facts that TTV mutates at a high frequency, it is found in many different types of cells and its genome has to undergo at least three separate replication steps in order to duplicate its original ss DNA viral genome, we hypothesized that g1- and g2-TTV viruses do not replicate independently of each other. This evidence indicates that g1- and g2-TTV viral genomes may interact so closely that DNA crossing over or DNA insertion from one TTV virus to the other is

likely. This could occur following viral penetration into the cell, during DNA replication (3 separate opportunities) or prior to viral assembly (after DNA replication has occurred). If both TTV genogroups are in close proximity and performing similar tasks it is not only likely but probable that there is some integration or genome sharing between the two viruses.

The Geneious software was revisited to compare the viral sequence obtained from the 3D4/2 cell line to known sequences of both g1- and g2-TTV. After only comparing a few known g1- and g2-TTV sequences deposited in GenBank to the isolated TTV sequence found in the 3D4/2 cell line an 81% match was found in which the isolated TTV DNA matched nucleotides with either known g1- or g2-TTV genomes. This could indicate that 81% of the isolated TTV virus came from either g1- or g2-TTV due to insertion or crossing over creating a hybrid g1-g2-TTV (fig. 7). However, 81% homology is still too low of a percentage to confirm a hybrid TTV has been created thus we must also consider the possibility of a novel undiscovered TTV which could fall into a new genogroup (g3?) and reacts with g1-TTV specific primers. Further analysis of the isolated TTV genome will be done by comparing the sequenced TTV to all known g1- and g2-TTVs or by isolating the original g1- and g2-TTV from the 3D4/2 cell line. There is also the possibility that the TTV present in the cell line is an undiscovered novel TTV that is not derived from g1-and/or g2-TTV.

We can now discuss the cloned g2-TTV viral DNA that is 99.5% homologous to a pig DNA sequence from clone CH242-347M1 on chromosome 12 (fig. 8). The cloned g2-TTV sequence

was not homologous to any known g1- or g2-TTV nucleotide sequences but matched almost exactly to 243 bp on chromosome 12. TTV has never been found to be imbedded in chromosomal cellular DNA. It was first thought that the primers from the PCR amplification step had amplified the wrong DNA segment and had actually amplified chromosome 12 DNA from the cell line due to poor amplification conditions. For this to occur the PCR primers may have bound to segments of chromosome 12 instead of g2-TTV viral DNA. This is a common occurrence in laboratory PCR because if the annealing temperature is too low then primers have the possibility of binding to an incorrect segment of DNA. With this in mind the cloned DNA sequence was revisited using the Genious software. The known sequence of chromosome 12 was searched and the exact g2-TTV primer sequence was found on the pig chromosome 12 indicating the PCR conditions did not cause the primers to bind to the wrong segment of DNA but in fact bound to the exact sequence of nucleotides expected based on the design of the primers.

This analysis could give rise to two possible conclusions. First, the g2-TTV DNA imbeds into the cellular chromosomal DNA. If this were the case, the primers would have bound to the complimentary nucleotide sequences on chromosome 12, and defined a portion of chromosome 12 for amplification, cloning, and then sequencing. If this were the case then the known pig chromosomal 12 DNA found in the Genbank archives must also have g2-TTV viral DNA imbedded in its genome. Assuming all of the above, is it possible that g2-TTV could be found imbedded in all porcine chromosome 12 DNAs? Another possibility to explain these

results is that the primers designed specifically for g2-TTV amplification are faulty and actually only bind and amplify a portion of the chromosome 12 DNA.

Figure 7

		1	10	20	30	40	50	60
	2843_F.ab1	CANAG--CTCCCGT-GAACACC-CNGCTC-A-NANGGAAACACGTGGTACCGGN---AAT						
2843_R.ab1	extraction 4 (reversed)	CAGAG--NTNCNGN-GAACACC-CAGNTC-A-AAGCGAAACANGGGGCAGNGAG---AAT						
	GU188046	TAGAGAACTCTCTA-AAATACCTCTGATT-AGACTAGGAATAAGCCTTAT--AG---AC-						
	AB049608 extraction 3	CA-ACAGCAGCCGTGGCTCGACTCCAGTCAAGAAGAGAAAAAGCGAGAGAGAGCAAAGC						
	2843_F.ab1	TCACTGNTACCGAATCCNANGTGGTGGGGGGGAATGACATTTGATGAATTCCTCCA						
2843_R.ab1	extraction 4 (reversed)	TCACNCAGCNNGAANNCAAGGGGGNGGGGGGAATGACATNNGATGAATTCCTCCA						
	GU188046	-CTCTCAGAACCATGGCTAGAAGG-GTGGGGAAATG-CATTTTAC-AGTGTCCTCGG						
	AB049608 extraction 3	CCAGGAAGAA-GAAGCCAAAACGCTGGAGCAGCA--ACTCGAGCAGCAGCGCCTCCT						

- 2843\_F.ab1 and 2843\_R.ab1 correspond with the forward and reverse sequences of the isolated TTV DNA from cell line 3D4/2. GU188046 and AB049608 represent the known g2- and g1-TTV sequences respectively.

*Drummond AJ, Ashton B, Buxton S, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2010) Geneious v5.0, Available from <http://www.geneious.com>*

Figure: 8

	1	10	20	30	40	50
2843-2_F.ab1 extraction 4						
2843-2_R.ab1 extraction 4 (reversed)						
CU914346						
2843-2_F.ab1 extraction 4	GACACTCAAGGCAAGGCTGTATTCTGTGGTCGGCGTGTCTCTCCTGTGACGGTCACCC					
2843-2_R.ab1 extraction 4 (reversed)	GACACTCAAGGCAAGGCTGTATTCTGTGGTCGGCGTGTCTCTCCTGTGACGGTCACCC					
CU914346	GACCCCTCAAGGCAAGGCTGTATTCTGTGGTCGGCGTGTCTCTCCTGTGACGGTCACCC					
2843-2_F.ab1 extraction 4	AAGAGTGGCTTTGGTCGCTGCAGTTTTAGAGAGGAGGGGTTGCTGGGACGTTTTGTGCA					
2843-2_R.ab1 extraction 4 (reversed)	AAGAGTGGCTTTGGTCGCTGCAGTTTTAGAGAGGAGGGGTTGCTGGGACGTTTTGTGCA					
CU914346	AAGAGTGGCTTTGGTCGCTGCAGTTTTAGAGAGGAGGGGTTGCTGGGACGTTTTGTGCA					
2843-2_F.ab1 extraction 4	CCCCACGGCCACGGGGGACGGCTTCCCGGAGCTGTCTGTGCCTCTGAGCTGCAGCGCA					
2843-2_R.ab1 extraction 4 (reversed)	CCCCACGGCCACGGGGGACGGCTTCCCGGAGCTGTCTGTGCCTCTGAGCTGCAGCGCA					
CU914346	CCCCACGGCCACGGGGGACGGCTTCCCGGAGCTGTCTGTGCCTCTGAGCTGCAGCGCA					
2843-2_F.ab1 extraction 4	TACCCGAGGGGTGTCTGCAGGCCTCGACGGACGTGTGCGCGAGTGTCTCTGCTGTAGCT					
2843-2_R.ab1 extraction 4 (reversed)	TACCCGAGGGGTGTCTGCAGGCCTCGACGGACGTGTGCGCGAGTGTCTCTGCTGTAGCT					
CU914346	TACCCGAGGGGTGTCTGCAGGCCTCGACGGACGTGTGCG-----					
2843-2_F.ab1 extraction 4	ACGGCACCACCGGCCACCCGTGCAAAACCTGCCATTGCTGTGCGCACTTACTAAGGGC					
2843-2_R.ab1 extraction 4 (reversed)	ACGGCACCACCGGCCACCCGTGCAAAACCTGCCATTGCTGTGCGCACTTACTAAGGGC					
CU914346	-----					
2843-2_F.ab1 extraction 4	AATTCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCG					
2843-2_R.ab1 extraction 4 (reversed)	AATTCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCG					
CU914346	-----					

- 2843-2\_F.ab1 and 2843-2\_R.ab1 correspond with the forward and reverse sequences of the isolated g2-TTV DNA from cell line 3D4/2. CU914346 represents the known sequence of chromosome 12 which is homologous to the cloned DNA from cell line 3D4/2.

*Drummond AJ, Ashton B, Buxton S, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S,*

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## CHAPTER 2: In Vivo Infectivity of 3D4/2-origin TTVs

**Section 2.1: Overview:** In virology, *in vivo* infectivity is a crucial characteristic when it comes to experimentally characterizing infectious viruses. When it comes to studying a certain virus the main goals are to identify the virus's characteristics, what symptoms the virus may cause and how to prevent the reproduction or spread of the virus through the use of anti-virals or vaccines. *In vivo* infectivity is necessary in order to help answer all of these questions.

TTV has not been successfully propagated *in vitro* making it almost impossible to identify the characteristics and symptoms associated with a TTV infection. Thus, identification of porcine cell lines that are persistently infected with porcine TTVs circumvents this major obstacle to virologic characterization of the TTVs. By successfully infecting gnotobiotic pigs with the TTVs that have replicated *in vitro* in 3D4/2 cells, TTV can be observed in a laboratory controlled environment. By infecting gnotobiotic pigs in a sterile environment, outside variables can be eliminated and any uncommon results regarding the pig's ability to potentiate and survive can be attributed to TTV infection. Once a list of common symptoms can be identified from the *in vivo* infection it will be much easier to diagnose the disease and also have a better understanding of its potential outcomes. *In vivo* infection can also give insight to how the torque teno virus reproduces inside of its host and is transmitted from one host to the next. With this information, vaccines and antivirals can be created in order to prevent the spread of disease. Successful *In vivo* infection will not only give us valuable information about TTV but will also provide the base for future studies and protection from the virus. This is why our goal in this experiment is to successfully infect gnotobiotic pigs.

## Section 2.2: Procedures

**GNOTOBIOTIC PIGLETS AND EXPERIMENTAL DESIGN:** Six piglets were derived into a sterile environment on March 16, 2011 by Caesarian section. Fourteen days later (March 30, 2011) three of the piglets (Piglet IDs of 11-529, 11-530, and 11-531) were infected with 3.0 mL of porcine alveolar macrophage (3D4/2) cells containing g1- and g2-TTV viral DNAs. The other three piglets (Piglet IDs of 11-527, 11-528, and 11-532) were uninfected and therefore considered controls. The gnotobiotic pigs were terminated 35 days after derivation (April 19, 2011). Pathologic evaluation of piglet tissue was conducted for me by my senior thesis advisor, Dr. Steven Krakowka. Once the TTV DNA levels in sera and tissue were determined by qPCR using TTV g1 and g2 specific primers, they were cross-referenced with the health of each piglet.

**TTV CHALLENGE INOCULUM (Same as in Section: 1.2):** The pigs were infected with a cell line designated: 3D4/2 containing g1- and g2-TTV viral DNAs. These cells were obtained from the American Type Culture Collection (ATCC), catalog number: CRL-2845. Upon receiving the cells, lab technician, Susan Ringler, subcultured the cell line. Every 2 to 3 days the cell line was subjected to addition passes in order to prevent the cultures from becoming overcrowded and dying off and to make additional cultures for future research. The cells were grown in RPMI-1640 medium with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum. The cell line was from the organism *Sus scrofa* (pig). It originated from lung macrophages



(alveolar) and had been transformed with pSV3-neo (Weingartl, 2002). The cells were propagated at 37°C in a 5% CO<sub>2</sub> Incubator.

**TISSUE SAMPLE COLLECTIONS:** Samples of left and right superficial inguinal and axillary lymph nodes, bronchial and mesenteric lymph nodes, thymus, lung, liver, spleen, bone marrow were collected into sterile vials and snap-frozen at –70C for future analysis of TTV DNA copy numbers by qPCR assay. Replicate samples of these tissues were collected into 100% (v/v) cold (4C) ethanol for 24 hours and then processed into paraffin blocks. Five micron replicate sections of each block were de-paraffinized, rehydrated, stained with hematoxylin and eosin, mounted with coverslips and examined by light microscopy. Histologic changes were noted and the severity of each was scored using a qualitative scale wherein 0 = no change from normal, 1 = minimal change from normal, 2 = moderate change from normal and 3 = severe change from normal.

## **Section: 2.3 – Results**

The 3D4/2 porcine alveolar cell line was used to infect gnotobiotic pigs. The cell line was subjected to qPCR using g1- and g2-TTV primers in order to discover if TTV was already present in the ATCC cell line. Each subculture showed that both g1- and g2-TTV was already present in the cell line. In addition porcine circovirus type 2 (PVC2) DNAs were also found in the cell line. Subculture number 4 was chosen for pig infection and contained  $4.97 \times 10^1$

copies/ng total DNAs. The cells underwent a freeze/thaw procedure prior to infection but were not clarified.

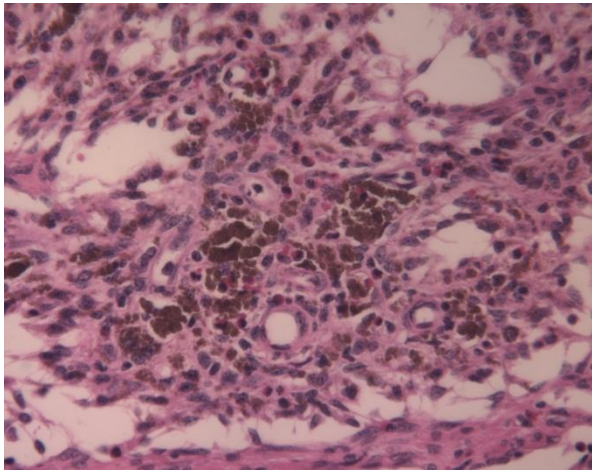
The pigs were derived on March 16, 2011 using Institutional Animal Care and Use Committee (IACUC) gnotobiotic derivation procedures. The piglets were kept in a sterile environment and were fed three times a day using the *Permalot* diet. At ten days of age, blood was drawn from each of the six pigs and qPCR was run on the samples using g1- and g2-TTV specific primers to determine if TTV DNAs were present, indicating prior *in utero* infection with one or both porcine genogroups. All sera were g1- and g2-TTV DNA-negative. Three piglets (11-529, 11-530, and 11-531) out of the six pigs were then infected with 3.0 mL of the 3D4/2 cell line 14 days of age. The other three pigs (11-527, 11-528, and 11-532) were untreated and considered controls. The pigs were observed for 20 days and terminated on 4-19-2011. During the observational period all three infected piglets developed were identified to have had iron-deficient anemia related to the *Permalot* diet that lacks colloidal iron dietary supplement. Piglet 11-529 exhibited symptoms of weakness and failure to thrive and was terminated 8 days after infection. The anemia of piglets 11-530 and 11-531 was then corrected by iron injections



**Figure 9: Brown lymph nodes indicating presence of hemosiderosis**

and they continued to the end of the experiment. After termination each pig underwent pathologic evaluation by Dr. Steven Krakowka; tissues were used to determine if infection did occur. In these evaluations the tissues used were: inguinal, axillary, mesenteric, bronchial, lung, thymus, liver

and kidney. Histologic findings were recorded and scored with a 0, 1, 2 or 3; 0 meaning no change from normal; 1 meaning minimal change from normal; 2 meaning moderate change from normal; 3 meaning severe change from normal (tables 1 and 2). Some histologic findings of note include: brown lymph nodes (fig. 9) and mild lymphofollicular hyperplasia found in all three pigs, Liver sections from 11-530 and 11-531 showed mild periphery-lobular hepatocyte degeneration and some level of hypoxia (fig. 10), and pigs 11-530 and 11-531 exhibited apical lobe depression and hemosiderin-laden lymph nodes. Further histological analysis by Dr.



**Figure 10: Brown discoloration of cells indicates hemosiderosis**

Steven Krakowka of each abnormal tissue sample was conducted (Tables 4, 5 and 6).

## **Section: 2.4 – Discussion**

Despite the iron-deficient anemia prevalent in all three challenged pigs during experimentation two pigs (11-530 and 11-531) were g1-TTV-DNA viremic after termination, indicating that that the 3D4/2 inoculum that contained g1- and g2-TTV-DNAs by qPCR contained infectious g1-TTV. This was determined by performing gel electrophoresis on post-terminal pig serum g1-TTV DNA bands were found in the challenged piglets while there was no band in the control serum. This gel electrophoresis test confirms that the 3D4/2 cell line containing TTV contained pig-infectious virus and induced a positive TTV DNA viremia status in these piglets. Thus, the primary goal of this pilot experiment that was performed to determine if the 3D4/2 cell line contains infectious porcine TTVs was accomplished.

However, upon analyzing the histological findings there was little or no change to most of the tissues in question. How then did TTV affect these piglets and why were there few indications of viral infection in the gross findings? There are several possible scenarios that would explain the absence of histologic changes in infected piglets. First, the challenged pigs were terminated 10 days after infection. All viruses are unique and vary greatly in the amount of time it takes for full infection to occur. In this case it is possible that not enough time was allowed for full TTV infection. Previous studies of TTV infection in gnotobiotics indicate that histologic lesions (exudative and interstitial pneumonia and membranous glomerulonephropathy) develop after post-infection day 14 (Krakowka, 2008). If this were the case then minimal histologic changes would be expected. This is easily tested by repeating the experiment and allowing the challenged piglets to live longer before termination. Another possibility for the minimal gross findings is the fact that TTV genotype(s) found in the 3D4/2 cell line could be infectious but avirulent in pigs. If this were the case then the TTV would be able to successfully infect the piglets but no disease would occur. This could be a result of prolonged *in vitro* replication within 3D4/2 cells, the virus may have lost intrinsic virulence properties but not its ability to infect swine. Finally, it may be that the TTV contained within 3D4/2 cells is sufficiently unique to be classified as a new genogroup (genogroup 3) of TTV. In humans, not all human TTV genogroups are virulent. Complete sequencing of recovered virus will be necessary before we can conclude that the 3D4/2 virus is sufficiently different from porcine g1- and g2-TTV to merit classification as a new porcine genogroup of TTV. While trying to understand the characteristics of TTV this would be a crucial finding. For example if only certain varieties of TTV infected specific species it would be possible to classify each TTV and its

specific host. This would then in turn lead to uniquely engineered vaccines specific for each species vulnerable to TTV infection and disease.

Regardless, the fact that 3D4/2-origin porcine TTVs are infectious for gnotobiotic swine represents an important new observation in TTV biology. Exhaustive studies of the TTVs are hampered by the conspicuous lack of methods for *in vitro* propagation of TTVs. This work establishes and characterizes a porcine TTV that replicates *in vitro* yet retains infectivity for swine. These data suggest that TTV-negative clones of 3D4/2 cells could well support infection by well characterized g1-TTV genotypes that have been used previously to establish the infectivity and pathogenic potential of the TTVs in pigs (Krakowka, 2008). Further studies with 3D4/2 TTV virus infection in gnotobiotic swine are warranted.

**Table 1**

Litter I.D.: 3-11 B					
Date of Birth: 3-16-11					
	Agent Administration	Infection	Sacrifice Date	Remarks	
Path # 11-527	No Treatment	--	4-19-11 @ 0910 with 1.5 cc Euthol IC	Inguinal: 0 axillary: 0 Mesenteric: 0 Bronchial: 0	lung: normal +1 thymic atrophy Notes: yellow brown lymph nodes
Description: Female White with Black Spots Weight (gm): 3080					
Path # 11-528	No Treatment	--	4-19-11 @ 0945 with 1.5 cc Euthol IC	Inguinal: 0 axillary: 0 Mesenteric: 0 Bronchial: 0	lung: normal thymus: normal Notes: brown lymph nodes
Description: Male White no notch Weight (gm): 3250					
Path # 11-529	TTV-3.0 mL IP (TTV in utero-negative)	3-30-11	4-19-11 @ 0930 with 1.5 cc Euthol IC	Inguinal: -- axillary: -- Mesenteric: 0 Bronchial: 0	lung: -- Notes: Terminal serum very watery, stomach distended with milk; apparent blockage, heart enlarged and pale liver
Description: Female Weight (gm): 1900					
Path # 11-530	in utero TTV negative TTV 3.0 mL IP	3-30-11	4-19-11 @ 1110 with 1.5 cc Euthol IC	Inguinal: 0 axillary: 0 Mesenteric: 0 Bronchial: +1	lung: -- Notes: Cassette #2 left apical lobe with depressed area near edge, brown nodes
Description: Female Right ear notch Weight (gm): 3020					
Path # 11-531	in utero TTV negative TTV 3.0 mL IP	3-30-11	4-19-11 @ 1200 with 1.5 cc Euthol IC	Inguinal: 0 axillary: 0 Mesenteric: 0 Bronchial: 0	lung: -- Thymic atrophy: +1 Notes: Cassette #2 apical lobes with depressed areas, brown nodes
Description: Male Spots with red rump Weight (gm): 3390					
Path # 11-532	No Treatment	--	4-19-11 @ 1015 with 1.5 cc Euthol IC	Inguinal: 0 axillary: 0 Mesenteric: 0 Bronchial: 0	lung: normal thymus: normal
Description: Male Right ear notch Weight (gm): 3220					

Gross changes scored as: 0 = no change from normal, 1 = minimal change from normal, 2 = moderate change from normal and 3 = severe change from normal

**Table 2.** A summary of histologic findings in three 14 day-old TTV-DNA viremia-negative gnotobiotic piglets infected with CD4/31 cells containing g1- and g2-TTV DNAs.

Group	Bronchial					Inguinal, Axillary													
	Lymph Nodes					Mesenteric					Thymus	Lung	Liver	Kidney					
						Lymph Nodes													
Tmt and	RE	GC	T-dp	T-pr	Syn	RE	GC	T-dp	T-pr	Syn	RE	T-dp	ly/pl	gran	ly	gran	emh	ly/pl	MGN <sup>a</sup>
Piglet ID																			
11-529 <sup>b</sup>	+/- <sup>c</sup>	0	-	0	-	1-2 <sup>d</sup>	0	-	0-1	-	not done	0	0	0	0	0	0	-	-
11-530	1	1	-	1	-	0-1	0-1	-	0-1	-	0	0	0	0	1	0	+/-	-	-
11-531	1	1	-	2	-	1	0-1	-	0-1	-	not done	0	0	1	0	+/-	-	-	-

<sup>a</sup> = abbreviations as follows: RE = reticuloendothelial (macrophages and dendritic cells) hyperplasia. GC = germinal center (B-cell dependent structures), T-dp = T cell depletion in the the lymph node paracortex, T-pr = T cell proliferation in the lymph node paracortex, Syn = syncytial giant cells, ly/pl = lymphocytic and plasmacytic cellular infiltrates, gran = granulomatous inflammation, emh = extramedullary hematopoiesis, MGN = Membranous glomerulonephropathy.

<sup>b</sup> Piglet 11-529 was terminated “early” due to weakness and failure to thrive.

<sup>c</sup> histologic changes subjectively scored as: (-) = not present; 0 = normal; +/- = possible change from normal; 1 = minimal change from normal; 2 = moderate change from normal and; 3 = severe change from normal.

<sup>d</sup> Macrophages in all lymphoid tissues contained hemosiderin, indicative of previous iron deficiency anemia due to diet.

**Table 3.** A summary of gross findings in three 14 day-old TTV-DNA viremia-negative gnotobiotic piglets infected with CD4/31 cells containing g1- and g2-TTV DNAs.

Group				icterus	thymic	bronchial	generalized	gross	gross lesions,
Tmt and	M/F	wt	term.	ascites	atrophy	lymphadeno-	lymphadeno-	liver	other organ
Piglet ID		(gms)	(PID)	(edema)		pathy	pathy	lesions	systems
11-529 <sup>a</sup>	F	1900	22	mild	0 <sup>a</sup>	0	0	pale	thin watery blood
			PID 8	edema					(Fe-def anemia)
11-530	F	3020	35	0	0	1	0	0	hemasiderin-laden
			PID 19						lymph nodes
11-531	M	3590	35	0	1	0	0	0	hemasiderin-laden
			PID 19						lymph nodes

<sup>a</sup> Piglet 11-529 was terminated “early” due to weakness and failure to thrive. All three piglets had iron-deficient anemia due to the new diet (*Permalat<sup>R</sup>*) that is deficient in iron. This was corrected by iron injections and the other two piglets continued to the end of the experiment. All three piglets had brown lymph nodes and lymphofollicular hyperplasia attributable to hemosiderin accumulations in macrophages.

<sup>b</sup> Gross changes scored as: 0 = no change from normal, 1 = minimal change from normal, 2 = moderate change from normal and 3 = severe change from normal.

TTV status: In spite of the preventable iron-deficiency anemia, this experiment was a success in that both piglets (11-530 and 11-531) were g1-TTV-DNA viremic at termination indicating that the porcine CD4/31 cell inoculum that contained g1- and g1-TTV-DNAs by PCR were shown to contain infectious g1-TTV (at least).



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